New Developments in Mutagenicity Screening Techniques with Yeast

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Introduction

Yeast as an Organism for Genetic Studies

Yeasts provide a useful and versatile tool for studying genetic phenomena. The genetics of *Saccharomyces cerevisiae* has been intensively studied for many years, and as a result considerable information has been accumulated. Excellent current and comprehensive reviews on the genetics of yeast are available *(1, 2)*, including one that is particularly directed toward studies of deleterious genetic effects of chemicals *(3)*. Yeasts are eukaryotic organisms and therefore contain a nucleus and cytoplasm containing various other differentiated organelles quite similar to other higher life forms *(4)*. The genetic apparatus consists of at least 17 chromosomes in the haploid *S. cerevisiae* identified by genetic mapping *(5)*. Thus the structural organization of the genetic information is analogous to that in other higher life forms. Equally important is the well-known life cycle in yeast which, genetically, provides for classical mitotic and meiotic functions such as those existing in differentiated cells of more complex multicellular organisms. Yeasts, as well as all higher life forms, contain extrachromosomal DNA which provides essential genetic information exclusive of the classical Mendelian pattern. *S. cerevisiae* is a particularly well-suited organism for the investigation of this little understood phenomenon.

In addition to the basic biological nature of yeasts, a number of practical considerations make these organisms particularly amenable for studies of the potential effects of chemicals on genetic events. Yeasts are routinely handled by using standard microbiological techniques such as cloning, replica plating, and micromanipulation. They grow rapidly, having short generation times, on inexpensive culture media, permitting accumulation of more than 10^9 cells from a single Petri dish culture within 2–3 days. On such media yeast divide mitotically by budding, which results in single isolated cells; therefore, the chance of a genetic change going undetected is minimized. There are numerous genetic markers available, including biochemical lesions, repair functions, suppressors, and regulatory functions, many of which are useful for studying chemically induced genetic effects *(1, 6, 7)*. The organism can be cultivated as a haploid, permitting mutation induction studies unhampered by considerations of the dominance or recessive nature of the mutation. Stable diploids and even higher ploidy can be readily constructed for studies involving such events as mitotic crossing-over and gene conversion. The mitotic and meiotic cycles can be interchanged in diploid *Saccharomyces* merely by changing the culture conditions.

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In summary, yeasts are practical and versatile organisms in which the effects of chemicals can be studied for their impact on the range of known genetic phenomena occurring in all eukaryotic life forms.

Some Current Concepts of Genetic Safety Screening

Geneticists interested in the problem of chemical mutagenesis have become increasingly aware that the metabolic activities in the animal have great impact on the fate of many potentially deleterious chemicals. Microorganisms apparently lack the requisite enzyme systems to metabolize many potentially genetically active compounds, and as a result such tests are negative. The issue has been clouded, therefore, by earlier studies involving microorganisms as the genetic indicator system.

In cases where the proximally active metabolites have been discovered, isolated, and then tested in microorganisms, they have been shown to cause genetic damage. Thus, in many instances, direct testing on genetic systems in microorganisms provides insufficient evidence to conclude that a compound is genetically harmless. Current methodology has brought us to the point where we can surmount these metabolic deficiencies in microorganisms. As indicated above, the genetically active breakdown product, if it is defined and reasonably stable, can be tested directly in the microorganism. Additional systems providing metabolic activity namely; (1) *in vitro* hydroxylation, (2) liver microsome preparations, and (3) host-mediated assays, will be described in this communication with special note to their applicability to *S. cerevisiae* genetic systems.

In our laboratories several specific lines of investigation are currently being pursued. These include forward and reverse mutation studies in haploid as well as reciprocal and nonreciprocal mitotic recombination in diploid *S. cerevisiae.* Since most of the chemicals whose genetic effects will be described in this paper are recognized carcinogens, the induction of deleterious genetic events in yeast may have relevance to the question to the mechanism of cancer induction.

In Vitro Genetic Assays

Qualitative Plate Tests: Forward and Reverse Mutation and Mitotic Gene Conversion

A significant proportion of the chemicals currently being screened for genetic activity are tested in qualitative plate tests. Procedures have been described for the detection of lambda phage induction (*8*) reverse mutation in *Salmonella typhimurium (9)* and *Escherichia coli (10)*, and mitotic gene conversion and reverse mutation induction in *S. cerevisiae* (*11–13*). Some serious limitations are associated with this approach to safety screening of chemical agents; for example, chemicals requiring metabolic activation prior to exhibiting genetic activity would appear negative in plate tests, as would compounds which have weak genetic activity. In addition, most of the systems in use have been built around the reversion of specific mutational alterations carried by the indicator organisms employed, which theoretically limits the types of compounds that can be detected in the assay to those capable of reverting the lesions in the indicator cells. Some technical modifications are being introduced in plate tests which should significantly increase their usefulness. These involve the utilization of hepatic drug-metabolizing enzymes for the potentiation of certain chemicals (*14*), construction of highly sensitive indicator strains through alterations in their capacity to repair genetic damage or limit permeability of specific molecules (*15*), and the development on nonrestrictive assays such as mitotic gene conversion (*11*) or forward mutation. An appropriate forward mutation test would permit the detection of all classes of mutagenic alterations.

A technique that we have been working with permits the detection of forward mutation at an arginine-specific permease locus in *S. cerevisiae.* A mutational block in this gene renders the cell resistant to an arginine

homolog, canavanine sulfate (*16*). The two-step plate test is conducted as follows. (1). Approximately 10^7 cells of the prototrophic haploid strain S228C are spread on plates of yeast complete agar (*17*) and allowed to dry. (2) an aliquot or a few crystals of the test chemicals are added to the plates. Solvent, if used, is added to the control plates. (3) Treated and control plates are incubated for 24 hr at 30° C. (4) Following this incubation, the plates are replica-plated to yeast minimal medium (*18*) containing canavanine sulfate (Calbiochem) at 30 μg/ml. (5) The canavanine plates are then incubated for 5 days at 30° C and scored for the number of canavanine-resistant colonies.

The advantages of this method are that apparently both frameshift and base-pair substitution events can be detected (*19*) and that the indicator organisms are exposed to the test chemical during several rounds of cell division, permitting the chemical to act on replicating DNA.

The results shown in Table 1 summarize our plate test studies with a large number of chemicals screened for forward and reverse mutation induction as well as for mitotic gene conversion.

The auxotrophic mutants used in the reverse mutation tests are classified as base-pair substitution (S211) and frameshift (S138) on the basis of extensive reversion tests with known frameshift and base-pair substitution mutagens.

Table 1. Results from qualitative plate tests detecting forward and reverse mutation and mitotic gene conversion.

Compound	Mitotic gene conversion [a b]	Forward mutation [b c]	Reverse mutation [d]	
			FS	BPS
MMS	+	+	±	+
EMS	+	+	−	+
MNNG	+	+	−	+
ENNG	NT	+	−	+
Diethyl sulfate	NT	NT	±	+
Nitrosomethylurea	NT	+	−	+
Nitrosomethylurethane	NT	NT	−	+
β-Propiolactone	+	+	±	+
Nitrogen mustard	NT	+	−	+
Triethylene melamine	+	+	−	+
Cyclophosphamide	−	−	−	−
DMNA	−	−	−	−
DENA	−	−	−	−
Hydroxylamine	−	−	−	−
Nitroquinoline *N*-oxide	NT	+	−	−
Hycanthone	+	−	−	−
ICR–170.E [e]	+	+	+	−
ICR–191.D [e]	+	NT	+	−
Sodium nitrite	NT	+	−	−
Quinacrine [e]	NT	−	−	−
Proflavine [e]	NT	−	−	−
2-Aminopurine	NT	−	−	−
5-Bromodeoxyuridine	NT	−	−	−

[a] MMS = methyl methanesulfonate; EMS = ethyl methanesulfonate; MNNG = *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; ENNG = *N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine; DMNA = dimethylnitrosamine; DENA= diethylnitrosamine; ICR–170 = 2-methoxy-6-chloro-9-[3-(ethyl-2-chloroethyl) aminopropylamino]acridine dihydrochloride; ICR–191 = 2-methoxy-6-chloro-9-[3-(2-chloroethyl)amino-propylamino]acridine dihydrochloride.

[b] Mitotic gene conversion at the *try* 5 locus in strain D–4.

[c] Forward mutation from canavanine-sensitive to canavanine-resistant in strain S288C.

[d] Reverse mutation in the *met*-auxotrophs S211 BPS (base-pair substitution) and S138 FS (frameshift).

[e] Compound requires testing medium to be adjusted to pH 7.0.

Mitotic Recombination: Crossing Over and Gene Conversion

Mitotic recombination (reciprocal crossing-over and non-reciprocal gene conversion) probably occurs in all eukaryotic diploid organisms. It has been observed in *Saccharomyces* (*20*), *Aspergillus* (*21*), *Penicillium* (*22*), *Ustilago* (*23*), *Drosophila* (*24*), and probably in mammals (*25,26*) including man (*27*). Many chemicals which are carcinogenic and mutagenic also induce mitotic crossing-over (*28–31*) and gene conversion (*11,32–37*). Thus chemically induced mitotic recombination can serve as an indicator of genetic damage (*38,11*).

The potential result of mitotic recombination is the generation of a new genotype by exchange of genetic material between homologous chromosomes followed by appropriate segregation of the products of mitosis. The consequence of such an event in a cell is the expression of a recessive phenotype in the homozygous condition which would oth-

erwise go undetected in the heterozygous state. Where carcinogenesis is shown to be a genetic phenomenon, mitotic recombination might serve as a mechanism to explain the origins of some tumors. The extended latent period commonly associated with tumor formation could conceptually be due to a heterozygous mutant "cancer gene" which is not expressed until later in life when mitotic recombination renders this gene homozygous. Mitotic recombination could likewise play a role in the aging process, as recessive homozygotes are continually and slowly generated in individuals throughout life.

A very simple system has been developed in *S. cerevisiae* strain D-3 for the detection of mitotic crossing-over or reciprocal recombination. A homozygous phenotype induced in the heterozygous diploid is the primary criterion for crossing-over. The *ade* 2 marker is particularly useful in this respect because the mutant allele results in the accumulation of a red pigment when homozygous but is recessive when heterozygous. Thus, mitotic crossing-over resulting in homozygosity of the *ade* 2 marker can be detected as a red sector in a normally white colony. Other genetic phenomena such as mutation or gene conversion could also result in expression of the recessive phenotype, so further testing is often desirable. With crossing-over, reciprocal homozygous products are obtained in each of the two daughter cells of mitosis. The particular diploid strain we use provides for this, since it has two flanking heterozygous markers around the *ade* 2 gene which can be tested in a limited population for induction of

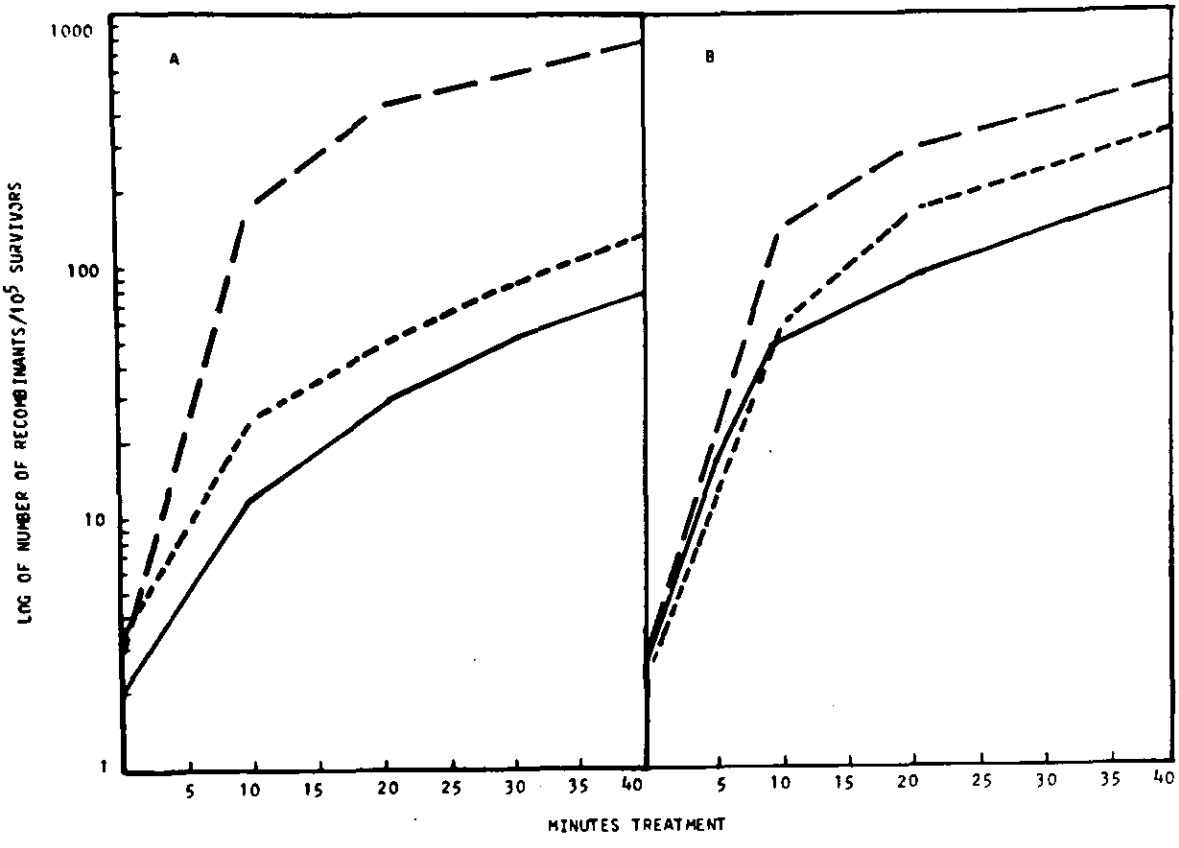

FIGURE 1. Induction of mitotic crossing-over and mitotic gene conversion in yeast by (——) MMS (0.1%), (—)$_6$ MNNG (25 μg/ml), and (--) EMS (5.0%): (*A*) strain D-4; (*B*) strain D-3.

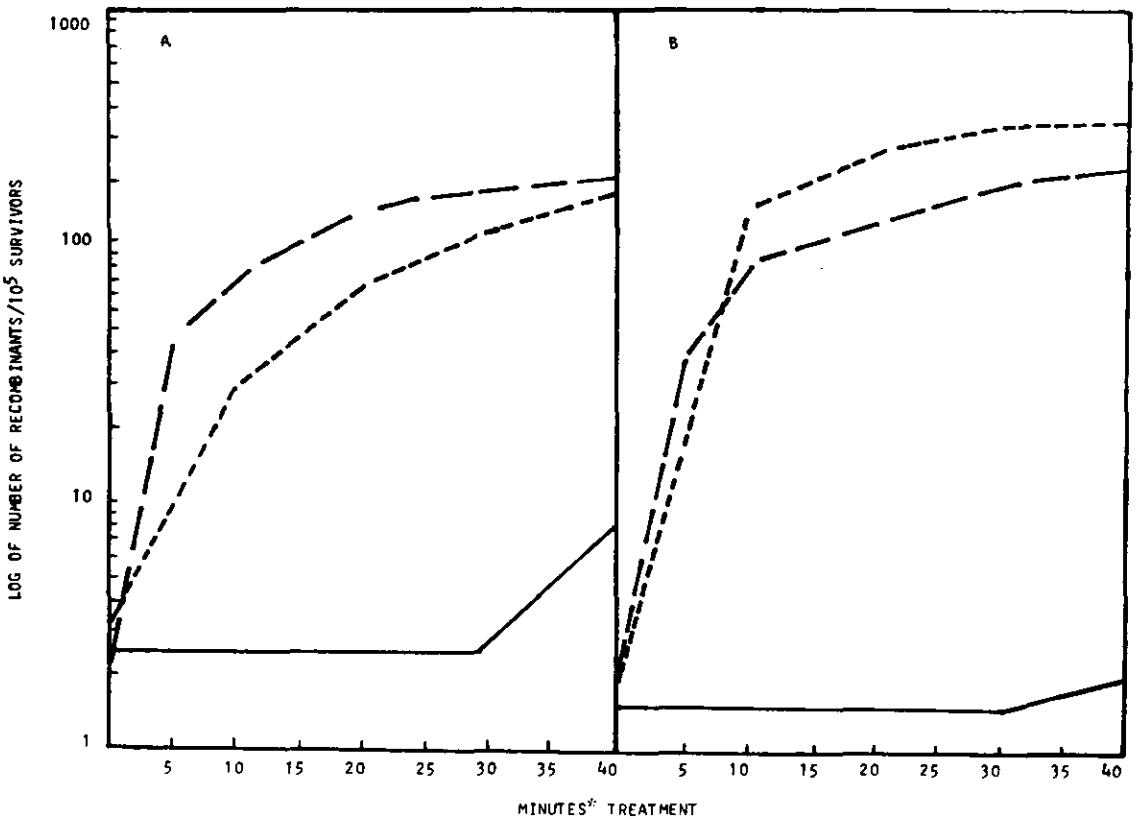

FIGURE 2. Induction of mitotic crossing-over and mitotic gene conversion in yeast by (——) hycanthone (1 mg/ml), (—) ICR 170 (10 μg/ml), and (--) ultraviolet light (10 ergs/mm^2 sec): (*A*) strain D-4; (*B*) strain D-3.

homozygosity in all three markers simultaneously.

A complete description of the particular strain and the methods for its manipulation and handling have been extensively described *(29–31)*. The details of the techniques used to obtain the mitotic crossing-over data presented in this paper are described by Mayer (*39*).

Mitotic gene conversion can be detected in strain D-4, a diploid strain of *S. cerevisiae* heteroallelic at the *ade* 2 and *try* 5 loci. These alleles are stable and show low frequencies of revertibility (*33*). Both heteroallelic loci result in nutritional deficiencies (noncomplementing) prohibiting the cells from growing on either minimal or single supplemented media. Mitotic gene conversion is a nonreciprocal event, probably involving alteration of a small number of nucleotide pairs within a single gene that can generate a wild-type allele at either of the two heteroallelic sites (*36*). This results in the expression of a functional gene and the loss of the nutritional requirement.

The results presented in Figures 1–3 and Table 2 compare the induction of mitotic recombination in the strains D–3 and D–4 under identical treatment conditions. Each curve, except those for hycanthone and cyclophosphamide, is the result of three independent experiments. The hycanthone results (Fig. 2 and Table 2) and the cyclophosphamide curves (Fig. 3) represent the results of two independent experiments each. Nonreciprocal recombination was assayed at both the *ade* 2 and *try* 5 sites of strain D-4 in all experiments. Since the re-

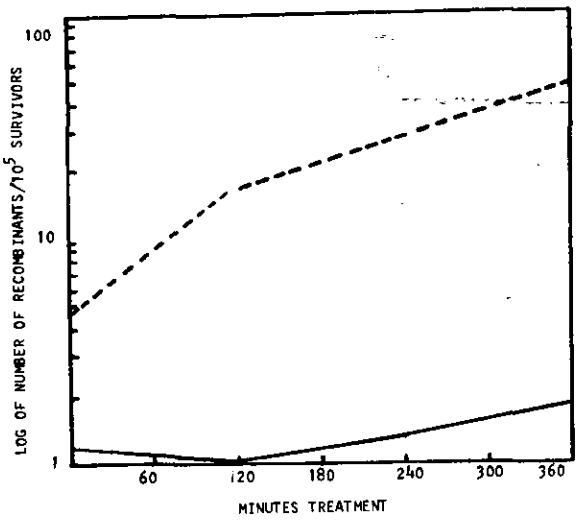

FIGURE 3. Induction of mitotic crossing-over and mitotic gene conversion in yeast by cyclophosphamide: (——) strain D-4; (--) strain D-3.

sults were not significantly different for the two sites and since the results were more consistent at the *try* 5 site, only these data were included in graphs or tables. To insure that the events detected in strain D-3 resulted from reciprocal recombination rather than some other lesion which might result in the production of red sectors (mutation, gene conversion, haploidization or nondisjunction), red and white portions of the resulting colony were analyzed for flanking markers *(29–31,39)*. In all of the cases that were studied, the results were consistent with the induction of reciprocal events.

Except for slight differences between ICR-170 and ultraviolet light (UV) (Fig. 2), the induction of recombination of both types are roughly the same for a single agent.

Three compounds, ICR-170, hycanthone, and cyclophosphamide, have not previously been reported to induce mitotic recombination *in vitro* and will be discussed in some detail.

ICR-170, 2-methoxy-6-chloro-9[3-(ethyl-2-chloroethyl)aminopropylamino]acridine dihydrochloride, is one of a series of potential antineoplastic compounds synthesized by Creech and co-workers (*40*). Many compounds of this series (including ICR-170) exhibit mutagenic activity in a wide range of organisms. ICR-170 is a potent recombinogen (Fig. 2) for both reciprocal and nonreciprocal events. ICR-191, 2-methoxy-6-chloro-9[3-(2-chloroethyl) aminopropylamino]acridine dihydrochloride, which is highly mutagenic for bacteria, and ICR-170 are also positive in the D-4 plate test (Table 1). Since these compounds are generally recognized as frameshift mutagens, it appears that mitotic recombination can be induced by frameshift as well as base-pair substitution mutagens. Fahrig (*41*) has also reported induction of nonreciprocal recombination in strain D-4 with acridine dyes.

Derivatives of hycanthone are potent antishistosomiasis drugs which have been shown to be mutagenic in bacteria (*42*), yeast (*43*), *Drosophila* (*44*) and animal cells (*45*). Cytological and teratogenic effects have also been reported for some of these derivatives (*46,47*). The results shown in Figure 2 indicate that this compound has a weak but significant recombinogenic activity, especially for D-3. In several experiments with hycanthone we observed that its genetic activity was time- and pH-dependent to a certain degree. A concentration of 1.0 mg/ml at pH 7.0 gave extreme cytotoxicity and only weak genetic activity; therefore, two lower doses were tested over a 4-hr incubation at pH 7.0 (Table 2). These tests confirmed the recombinogenic activity of hycanthone for the reciprocal recombination but left serious doubts as to its efficacy for the induction of nonreciprocal events. Because hycanthone did produce a positive response in the D-4 plate test, we are conducting additional tests to determine what environmental factors are required to obtain an accurate assessment of the activity of this agent in D-4.

Cyclophosphamide (Cytoxan) is used chemotherapeutically against many types of tumors and metabolized by the mixed func-

Table 2. Induction of mitotic recombination in strains D–3 and D–4 with hycanthone at concentrations of 0.01 and 0.10 mg/ml.

Treatment	Survival, %		Recombinants/10^5 Survivors	
	D–3	D–4	D–3	D–4
Control	100	100	12	5
Hycanthone, 0.01 mg/ml	67	100	26	3
Hycanthone, 0.10 mg/ml	32	67	92	6

tion oxidase enzymes of the liver to a cyclic nitrogen mustard (*48*). Chromosome damage (*49*) and recombinogenic activity by a urine-excreted metabolite (*50,51*) have been reported for this chemical. The results shown in Figure 3 indicate that a significant recombinogenic activity in D-3 is observed with cyclophosphamide *in vitro*. No induction of nonreciprocal events was observed in the *in vitro* tests which is consistent with other published observations (*50, 51*). The potent activity of this compound for the induction of mitotic recombination in strain D-3 without previous metabolic activation was unexpected, and a more detailed study of this compound is currently under way.

In Vitro Hydroxylation System

Dimethylnitrosamine (DMNA) and diethylnitrosamine (DENA) require conversion to alkylating agents for their activity, presumably through a mechanism involving an initial α-carbon hydroxylation followed by spontaneous dealkylation (*52–55*). Studies of aromatic amines indicate that they are carcinogenic only after metabolic conversion by a hydroxylation mechanism to active breakdown products (*56*). Preussmann (*57*) showed that aliphatic nitrosamines can be hydroxylated by an enzyme-free system that was originally developed by Udenfriend et al. (*58*) for the ring-carbon hydroxylation of aromatic compounds. Malling (*59,60*) and later Mayer (*61*) demonstrated the formation of mutagenic breakdown products from DMNA and DENA using this system with *Neurospora* and *Saccharomyces*, respectively. Recently, Locke and Mayer (*62*) demonstrated by physical and chemical methods that the Udenfriend system was indeed capable of dealkylation reactions. After the *N*-hydroxy derivatives of 1-naphthylamine (1-NA) and 2-naphthylamine (2-NA), but not the parent compounds, were found to be mutagenic for microorganisms (*63–67*), Mayer (*68*) demonstrated that the breakdown products of 1-NA and 2-NA formed in the Udenfriend hydroxylation system, but not the parent compounds, were mutagenic for *Saccharomyces*. More recently DMNA, DENA, 1-NA, and 2-NA breakdown products have all been shown to induce mitotic crossing-over in *Saccharomyces* using the Udenfriend hydroxylation system (*39*).

The research presented in this communication is an extention of these above findings, including further studies on aliphatic nitrosamines and aromatic amines. The methodology has been previously reported (*39, 61, 68*). Essentially, the procedures involve simply incubating the genetic indicator organism with the test chemical in the hydroxylation medium and removing cell samples at appropriate time intervals to assay for induced genetic events. Since the breakdown reactions require molecular oxygen, the effect of breakdown products can be measured in samples bubbled with O_2 gas. Similar samples bubbled with N_2 gas give results of testing the parent compound for genetic activity without breakdown.

Mitotic crossing-over was induced by DMNA, DENA, di-*n*-propylnitrosamine (DPNA) and di-*n*-butylnitrosamine (DBNA) in the Udenfriend reaction mixture in the presence of oxygen but not in the presence of nitrogen (Fig. 4). Since the reaction requires oxygen (*57,58*), these results indicate that the parent compounds are inactive but that some breakdown products are the genetically active entity. It is also noteworthy (Fig. 4) that DENA breakdown products induced crossing-over to a consider-

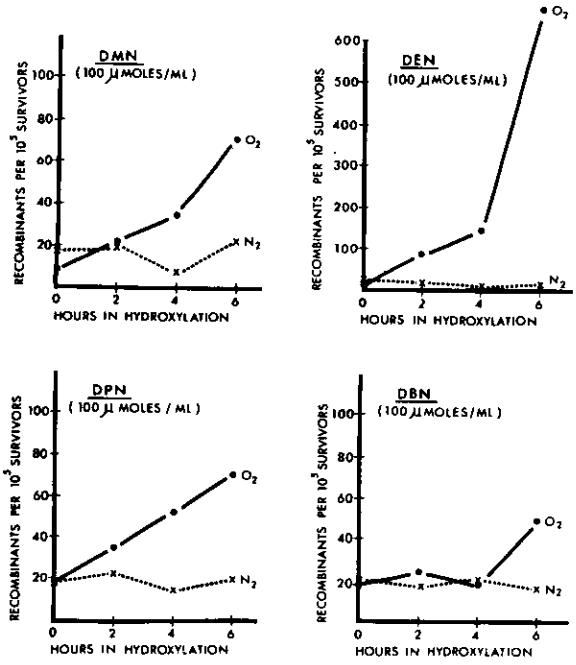

FIGURE 4. Mitotic crossing-over induced in strain D-3 by breakdown products of aliphatic nitrosamines formed in the Udenfriend hydroxylation medium.

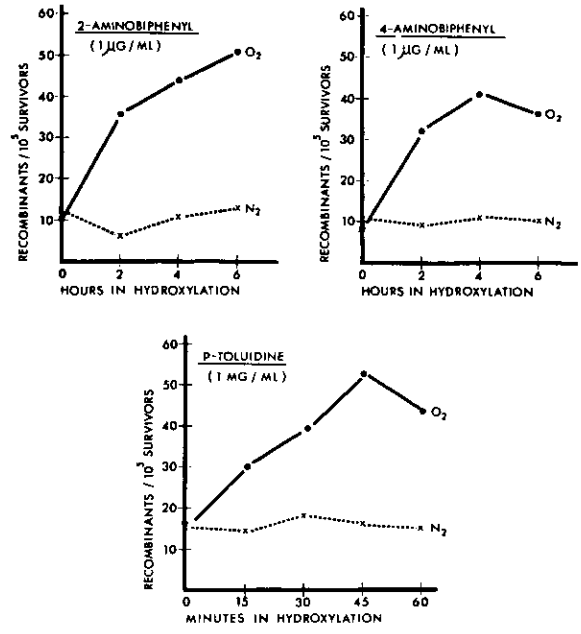

FIGURE 5. Mitotic crossing-over induced in strain D-3 by breakdown products of aromatic amines formed in the Udenfriend hydroxylation medium.

ably greater extent than did DMNA, DPNA, or DBNA. Likewise a relationship between sector induction and time of treatment is apparent for each compound. The homologous secondary amines, dimethylamine and diethylamine, which lack the nitroso group of DMNA and DENA, did not induce crossing-over under any test conditions (*39*). The fact that they are not mutagenic (*61*) indicates that the nitroso group is essential for the genetic activity of DMNA and DENA.

The induction of mitotic crossing-over by breakdown products of three aromatic amines, 2-aminobiphenyl, 4-aminobiphenyl, and *p*-toluidine are shown in Figure 5. Again, only samples of compound in the Udenfriend reaction medium with oxygen give responses above control values. Survival in all samples was 70% or higher. Other aromatic amines, 1-NA and 2-NA, were previously shown to induce mutation (*68*) and mitotic crossing-over (*39*) in *Saccharomyces* with the Udenfriend reaction medium only after breakdown in the presence of oxygen.

A representative sample of sectored colonies induced by 2-aminobiphenyl, 4-aminobiphenyl, and *p*-toluidine were cloned for their red and white portions. These were each tested for induced homozygosity of a flanking heterozygous marker on either side of the *ade* 2 gene. The simultaneous induction of homozygosity in these three markers was observed in the majority of samples, further indicating that induction of the red sectors by these chemicals was due to reciprocal crossing-over and not to mutation or gene conversion. Similar results have been reported for DMNA, DENA, 1-NA, and 2-NA (*39*).

In Vitro Liver Microsome Activation of Chemical Mutagens

Malling (*69*) demonstrated that the metabolites of DMNA could be formed by exposure of the compound to mouse liver

microsomal enzymes *in vitro* as well as in host-mediated or enzyme-free hydroxylation tests. Ames et al. (*14*) have also used rat and human liver microsomes to activate chemical carcinogens in bacterial plate tests. The addition of liver microsomal enzymes to *in vitro* assays has not only proven to be a convenient technique to screen large numbers of chemicals for genetic activity but is also a potentially valuable method for quantitatively comparing the metabolic capacities of different species, or individuals within a species, to convert a given compound to its genetically active state.

We have developed an *in vitro* liver microsome assay in our laboratory and have been using both bacteria and yeast as indicator organisms. Chemical carcinogens and mutagens have been activated by microsomes obtained from several rodent and primate species. The system uses the 9,000 *g* supernatant from liver homogenates prepared in 0.25*M* sucrose. A single run is conducted in a 50-ml prescription bottle with a final volume of 1.8 ml. This includes the reaction mixture, test chemical, microsome fraction and the indicator cells. A typical test with DMNA and strain D-3 is shown in Table 3. Pure O_2 is blown into the bottle, and it is then incubated at 37°C for 60 min lying on its side to provide maximum exposure of the fluid to the O_2. All dilutions and platings are the same as those used in host-mediated and normal *in vitro* tests.

The results of tests with DMNA and DENA are shown in Table 4. Neither of these compounds are recombinogenic *in vitro* without metabolic activation.

Table 3. *In vitro* liver microsomal assay with yeast as indicator organisms.

Component	Concentration, ml^{-1}
TPN (sodium salt)	6 μmole
Isocitric acid	49 μmole
Isocitric dehydrogenase	25 μl
$MgCl_2$	4 μmole
Tris buffer, pH 7.4	29 μmole
Microsome fraction	63 mg
DMNA	75 μmole
D–3 cells	3–5 × 10^7

Table 4. Recombinogenic properties of DMNA and DENA following *in vitro* activation by mouse liver microsomes.[a]

Treatment	Test compound concentration, μmole/ml	Recombinants per 10^5 survivors
Control	0	8.3
DMNA		
30 min	75	15.4
60 min	75	26.1
DENA		
30 min	100	9.1
60 min	100	20.5

[a] Microsome fraction was obtained from randombred Swiss Albino male mice.

Both compounds are converted to recombinogenic intermediates by the liver microsomes at concentrations significantly lower than those required to produce genetic activity in the HMA. It appears that DMNA exhibits greater recombinogenic activity in this assay than does DENA. Such is also the case in HMA tests with either bacterial or yeast cells as indicators. However, in the enzyme-free hydroxylation system, DENA shows greater genetic activity for *Neurospora* (*59,60*) and yeast (*61*) than does DMNA.

The Host-Mediated Assay

Utilization of *S. cerevisiae* in mouse host-mediated array (HMA) tests has been described (*70–72*), and a summary of the results from published literature is presented in Table 5. An abstract recently published by Fahrig (*73*) describes the testing of DMNA, MMS, 4-nitroquinoline 1-oxide, MNNG, and ICR–191 in HMA tests with strain D–4. The report does not make clear whether all of the compounds were positive or only some of them. In addition to the use of strains D–3 and D–4, we have experimented with haploid yeast strains in which forward and reverse mutation could be detected. The overall results indicated that the least strain specificity and the maximum degree of sensitivity could be obtained with the diploid strains.

Table 5. Summary of published host-mediated assay results with *S. cerevisiae* as the indicator organism.

Compound	Recombinogenic activity In D–3	In D–4	Reference
EMS	+	+	(*71*)
Cyclophosphamide	+	+*	(*71*)
Aryldialkyltriazines	NT	+	(*70*)

* Activity observed only when cells of D–4 were exposed to the urine of the treated host.

Table 6. Summary of host-mediated assay tests with *S. cerevisiae* strain D–3 as the indicator organism.

Compound	Active *in vitro*	Minimum effective dose, mg/kg	Response
EMS	+	150	+
MMS	+	100	+
DMNA	–	1300	+
DENA	–	1900	+
Cyclophosphamide	+	400	+
Hycanthone	+	—	none at 100 mg/kg

Specific protocols for the use of yeast cells in the HMA have been described elsewhere (*70,72*). In most respects, yeast cells serve quite well as genetic indicators in this technique. Their recovery from the peritoneum of the host animal is good, with essentially 100% viability among recovered cells. We have observed no change in the number of recovered D–3 cells during the course of a 4-hr experiment. This may indicate that little or no cell division occurs during the incubation or that there is a balance between growth and lethality maintaining a constant recoverable population. Good recoveries have been obtained up to 24 hr after injection, indicating that relatively long-term exposures are feasible. The injected yeast cells remain dispersed in the peritoneum and exhibit very little clumping. They can be diluted and plated immediately following removal from the sacrificed host.

Results of our yeast HMA tests with several compounds are summarized in Table 6. Compounds that are recorded positive showed either a dose response or were positive in at least five independent tests but did not demonstrate a dose response. The doses shown represent the minimum concentrations at which some positive (not necessarily significant) effects were observed. Generally three or four dose levels were employed for each chemical. The tests summarized in Table 6 were conducted in randombred Swiss Albino male mice with 3-hr exposure period. The peritoneal exudates from three mice were pooled before being diluted and plated. In current tests individual animals are analyzed and recorded independently. This method provides better analysis of the data and is preferred.

The results in the Table 6 illustrate that for most compounds high dose levels are required to produce detectable responses in yeast. This was also true for our tests with strain D–4. In fact, we have had consistently better results with strain D–3 than D–4. A comparison of the strains D–3 and D–4 with three compounds is presented in Table 7. The results show clearly that with similar concentrations of MMS, EMS and cyclophosphamide, strain D–3 appears to be more sensitive than D–4. It will be important to see how our results compare with Fahrig's data since many of the same compounds were tested in his laboratory and ours. Published reports by Siebert (*51*) also indicated that cyclophosphamide was not active in standard HMA tests but that urine from the treated animals was recombinogenic. The results in Table 7 were obtained from analysis of individual animals. Table 8 shows the results of a retest with hycanthone in which all test and control animals were assayed individually. The results are consistent with those of the earlier tests.

Summary

Several points should be emphasized regarding the use of yeast in genetic assays conducted as part of the safety evaluation of chemicals in relation to their risk in the human population.

Yeast provides a versatile eukaryotic organism to detect as well as evaluate chem-

Table 7. Comparison of the induction of reciprocal and nonreciprocal mitotic recombination in the host-mediated assay with MMS, EMS and cyclophosphamide.

Treatment	Animal no.	Dose, mg/kg	Reciprocal recombinants per 10^5 survivors		Nonreciprocal recombinants per 10^5 survivors	
3 Controls	1	0	0.8	7.4 [a]	1.3	2.0 [a]
	2	0	2.5	0.0	2.0	3.2
	3	0	0.5	7.6	1.1	2.7
	4	0	3.0	6.1	0.0	2.4
	5	0	4.0		1.7	2.0
		Mean	2.2	5.3	1.3	2.5
MMS	1	100	9.6		2.5	
	2	100	13.0		4.4	
	3	100	3.5		3.0	
	4	100	6.2		3.6	
	5	100	12.1		0.0	
		Mean	8.9		2.7	
EMS	1	350	23.0		1.8	
	2	350	14.2		0.0	
	3	350	5.3		2.5	
	4	350	11.4		2.1	
	5	350	24.7		2.3	
		Mean	15.7		1.7	
Cyclophosphamide	1	800		15.7		2.3
	2	800		13.6		2.2
	3	800		20.2		2.6
	4	800		8.0		2.6
	5	800		16.2		3.2
		Mean		15.1		2.6

[a] Controls for cyclophosphamide.

icals possessing mutagenic and recombinogenic activity. Most of the assays in which bacteria or other microorgansims are used as plate tests, host-mediated tests and *in vitro* microsome-activated assays can be modified to accommodate some haploid or diploid strain of yeast. This offers an ideal situation in which the activity of a particular compound can be compared to prokaryotic and eukaryotic cell types.

Yeast is uniquely suited for the study of mitotic recombination and gene conversion. A number of markers can be conveniently used for these studies, and the resultant products can be genetically analyzed to define the type of event that has occurred. Chemicals which can induce mitotic recombination may play a significant role in making homozygotic those pre-existing or recently induced recessive mutations in a diploid cell undergoing mitotic cell division, with the concomitant effects the expression of the homozygous alleles might exert on the developmental, aging and biochemical functions of the individual cell or organism in which the gene resides. There are sufficient data reported here and by other investigators to suggest that the induction of mitotic recombination exhibits very little or no chemical specificity and that these events are often induced at a lower concentration of the particular chemical than required for gene mutation induction.

The doses necessary to produce genetic effects in yeast indicator cells are quite high in many cases. For example, in the HMA, DMNA will induce a strong mutagenic response in *Salmonella* strain G–46 at a dose 100 times lower than that required to obtain an effect in strain D–3 of the yeast. This

Table 8. Effect of hycanthone in the host-mediated assay.

Treatment	Animal no.	Dose, mg/kg		Reciprocal recombinants per 10^8 survivors
Control	1	0		1.4
	2	0		1.2
	3	0		1.0
	4	0		1.0
	5	0		0.9
	6	0		0.9
	7	0		0.9
	8	0		0.8
	9	0		0.8
	10	0		0.7
			Mean	1.0
Hycanthone	1	100		1.3
	2	100		1.1
	3	100		1.1
	4	100		1.1
	5	100		1.1
	6	100		1.0
	7	100		0.9
	8	100		0.8
	9	100		0.7
	10	100		0.5
			Mean	1.0

low sensitivity in yeast compared to bacteria may result from a number of things, including differences in physical parameters such as cell walls or internal membrane barriers not present in bacteria. The differences might also reflect biochemical differences between the two cell types relating to enzymatic modification of the chemical or the number of susceptible target sites in the respective genomes. Obviously there exist qualitative and quantitative differences between the mutagenic sensitivity and specificity in pro- and eukaryotic cells, and until the mechanisms resulting in these differences are understood, it will be difficult to assess the relevance of a response obtained from a given indicator organism to the potential risk to human cells.

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